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Both the Fast and Slow Refolding Reactions of Ribonuclease A Yield Native Enzyme

(pH jump/stopped flow/inhibitor binding/tyrosine absorbance)

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ABSTRACT **The fast reaction ($\tau_2 \simeq 50$ msec) observed previously in the refolding of thermally unfolded ribonuclease A (disulfide bonds intact) has now been studied by two properties indicative of enzyme function: binding of a competitive inhibitor (2′CMP) and hydrolysis of a substrate (CpA → C > p + A). Both the binding and catalytic reactions are fast (<2 msec) compared to refolding. Binding of 2′CMP occurs during both fast and slow refolding reactions, and the protein folded in the fast reaction has a normal binding constant for 2′CMP. Recovery of enzymatic activity during the fast refolding reaction, as measured by the rate of CpA hydrolysis, parallels the kinetic curve for 2′CMP binding. When the kinetics of refolding are measured by the burying of exposed tyrosine groups, no difference is found. The presence of 2′CMP has no effect on the kinetics of refolding.**

The results show that the fast refolding reaction does not yield an intermediate in the refolding of RNase A. Instead, both fast and slow refolding reactions have a common product, fully active RNase A. Although they show a 100-fold difference in rates of refolding, the starting materials for the fast and slow refolding reactions have similar properties, as regards: (*a*) the molar absorbancy at 286 nm, reflecting the state of exposed tyrosine groups, and (*b*) their apparent failure to bind 2′CMP.

Both fast and slow refolding reactions have been observed in studies of the reversible unfolding transitions of a few simple small proteins: in the pH-induced refolding of staphylococcal nuclease (1) and bovine pancreatic RNase A (2) and in the solvent-induced refolding of horse-heart ferricytochrome *c* (3, 4) and chicken egg-white lysozyme (5) in guanidine solutions. The interpretation of these fast refolding reactions has been in doubt, but it has been supposed that they represent the formation of one or more intermediates in refolding: either intermediates on the normal pathway (1) or abortive intermediates, not on the direct pathway (3, 4). An alternative possibility is that the fast reactions represent the conversion of intermediates to final product. For RNase A, this may be tested by measuring the enzymatic activity of the product of fast refolding in a time range where the extent of slow refolding is small.

In previous work (2), the refolding of RNase A has been measured by the changes in absorbance at 286 nm or at 240 nm that result chiefly from shielding tyrosine groups from solvent. There are six tyrosine groups in RNase A. They are found in different regions of the three-dimensional structure (6, 7) rather remote from the active site, and all of the tyrosine groups are at least partly shielded from water at neutral pH, where they are not ionized (7). Titration experiments (8, 9) show that three of the six tyrosine groups are not free to ionize normally in native RNase A, and spectral studies (10, 11) also show differences between individual tyrosine groups. Spectral changes caused by burying tyrosine groups during refolding probably reflect conformational changes in more than one region of the RNase A molecule. Partial refolding might be observable via the tyrosine groups, whereas substrate binding and catalytic activity are likely to require complete refolding.

Abbreviations: RNase A, bovine pancreatic ribonuclease A; 2′CMP, cytidine 2′-phosphate; C > p, cytidine 2′,3′-cyclic phosphate; T_m, temperature midpoint of the thermal unfolding transition.

EXPERIMENTAL

Materials. (*a*) RNase A: Worthington lot no. RASE 2HB, filtered through Sephadex G-25 (Pharmacia) to remove phenol and phosphate; stored in 0.1 M $NaClO_4$; concentration measured by absorbance at 278 nm, neutral pH, using a molar absorbance of 9.8×10^3 (20). (*b*) 2′CMP: P. L. Biochemicals lot 273-10; concentration measured using a molar absorbance of 7.6×10^3 at 260 nm, neutral pH (21); the ratio A_{280}/A_{260} was found to be 0.84 [lit. 0.85 (21)]. (*c*) CpA: Sigma lot 30 G-7490; molar absorbance (266 nm) = 2.1×10^4 in 0.1 N HCl (22).

Buffers. (*a*) pH 5.8: 0.1 M $NaClO_4$, 0.05 M cacodylate. (*b*) pH 2.0: 0.1 M $NaClO_4$, sufficient $HClO_4$ to give pH 2.0.

Methods. (*a*) Equilibrium measurements of absorbance were made in a Cary 14 spectrophotometer with a water-jacketed cell holder; the temperature was measured by a dipping thermistor in the cell. (*b*) Kinetic measurements were made with a modified Gibson-Durrum stopped-flow instrument, using a Tektronix 564 storage oscilloscope. The temperature was controlled by water circulation. After degassing, solutions were transferred to the driving syringes by plastic disposable syringes (Stylex) but were not allowed to stay in contact with plastic for more than a few minutes. Long storage (30 min or more) of RNase A (pH 2.0) in the plastic syringes gave rise to new, slower, refolding reactions in which the refolded protein was enzymatically inactive.

RESULTS

Refolding of thermally unfolded RNase A goes to completion after a pH jump from 2.0 to 5.8. There is a substantial change in A_{250} when 2′CMP binds to native RNase A (12). [The change is believed to occur in the spectrum of the nucleotide, not that of the enzyme (13).] Binding is rapid [$k = 1 \times 10^7$ M^{-1} sec^{-1} at 15°, pH 6 (14)] and a specific 1:1 complex is formed between 2′CMP and native RNase A (12, 15). We find no difference spectrum when 2′CMP is added to thermally

TABLE 1. *Test for complete refolding of RNase A by 2'CMP binding*

Experiment*	ΔA_{250}†
(1) Native RNase A (47°, pH 5.8 + 2'CMP, 10 min)	0.185 ± 0.002
(2) Unfolded RNase A (47°, pH 2.0, 10 min) → Native RNase A (47°, pH 5.8 + 2'CMP, 10 min)	0.181 ± 0.002

* Final concentrations: RNase A, 0.90×10^{-4} M; 2'CMP, 1.35×10^{-4} M.

† ΔA_{250} is the difference between the value of A_{250} read after and before adding 2'CMP; the same amount of 2'CMP is added both to the sample and the blank, and A_{250} is read after 10 min (at pH 5.8, 47°) in a Cary 14 spectrophotometer.

unfolded RNase A [75° (pH 5.8)]. Thus ΔA_{250}, the change resulting from 2'CMP binding may be used to measure the completeness of refolding of thermally-unfolded RNase A after a pH jump from 2.0 to 5.8. Table 1 shows such an experiment. 10 min after adding equal amounts of 2'CMP to the blank and to a standard solution of native RNase A (pH 5.8), 47°, ΔA_{250} is read in a conventional spectrophotometer. The procedure is repeated with another aliquot of the same RNase A solution previously exposed to unfolding conditions at pH 2.0, 47°, for 10 min; the 2'CMP is added together with the concentrated buffer used to bring the pH from 2.0 to 5.8, and all samples are brought to the same final volume. Comparison of the two values of ΔA_{250} (Table 1) shows that refolding is at least 98% complete, that it yields native RNase A capable of binding 2'CMP, and that no very slow refolding reactions (compared to 10 min) contribute to the recovery of 2'CMP binding. Fig. 1 shows the thermal transition zones for unfolding measured by tyrosine groups at the initial pH of 2.0 and the final pH of 5.8. Comparison of the heating and cooling curves also shows good reversibility of unfolding measured in this way. At 47° we can study essentially complete refolding, with minimal complications from partly-folded intermediates that may be produced inside the transition zones at the initial or final pHs.

Binding of 2'CMP occurs during the fast refolding reaction. As expected from the second-order rate constant, binding of

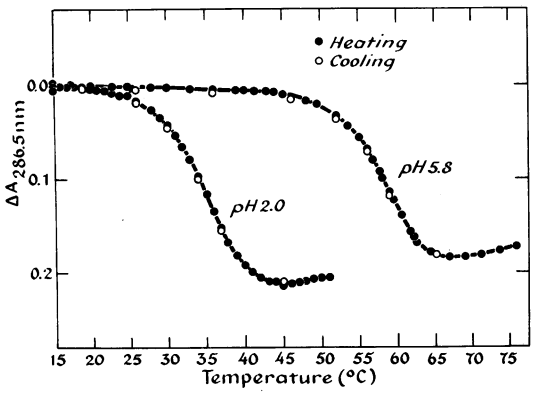

FIG. 1. The transition zones for thermal unfolding of RNase A in the initial (pH 2.0) and final (pH 5.8) conditions, measured by buried tyrosine groups via A_{286}. Concentration of RNase A: 0.92×10^{-4} M (see *Experimental* for buffers.)

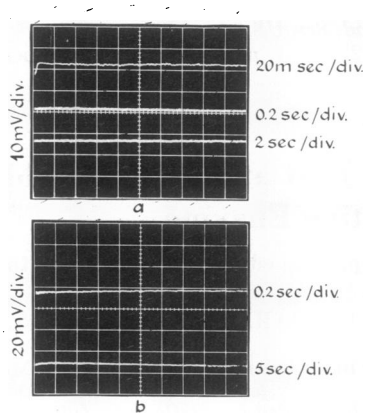

FIG. 2. Controls for the measurement of regeneration of the 2'CMP binding site during refolding of RNase A at pH 5.8. (*a*) After adding 2'CMP to native RNase A at pH 5.8, 27°, the reaction observed at 250 nm goes to completion within the stopped-flow dead time of 2 msec. Total signal 0.35 V. (*b*) In the absence of 2'CMP, refolding of RNase A (pH 2.0 → 5.8, 47°), causes only small changes at 250 nm. Total signal 2 V.

2'CMP to native RNase A is complete within the stopped-flow deadtime of 2 msec and there are no significant later changes in A_{250} in any of the time ranges used to study fast and slow refolding (Fig. 2*a*). The wavelength 250 nm has been chose because ΔA_{250} for refolding, in the absence of 2'CMP, is quite small (Fig. 2*b*) compared to ΔA_{250} for the binding of 2'CMP to native RNase A. Stopped-flow measurements of ΔA_{250} during refolding after a pH jump 2.0 → 5.8 + 2'CMP show that binding of 2'CMP occurs during both the fast phase (Fig. 3*b*) and the slow phase (Fig. 3*a*) of refolding. The relaxation times and amplitudes are closely comparable to previous values (2) obtained at pH 3.9 and 7.0 by studying tyrosine groups.

TABLE 2. *2'CMP binding in the fast and slow refolding reactions of RNase A (47°, pH 5.8)*

2' CMP (10^{-5} M)	r*	Δy† (fast) (mV)	Δy† (slow) (mV)	α_2‡	α_2 calc§
0		—	<5	—	
0.75	0.164	9	35	0.21	0.148
1.25	0.263	18	65	0.22	0.151
2.50	0.460	26	95	0.22	0.159
3.75	0.597	38	140	0.22	0.172
5.00	0.691	40	150	0.21	0.185
7.50	0.795	42	155	0.21	0.210

* Fraction of the RNase A sample binding 2'CMP at equilibrium calculated from a spectrophotometrically measured dissociation constant of 1.3×10^{-5} M at 47° and the RNase A concentration of 3×10^{-5} M. † Change in signal at 250 nm resulting from 2'CMP binding in the fast and slow refolding reactions, respectively; total signal 2V. ‡ Fraction of the total 2'CMP bound which is bound in the fast refolding reaction. § Calculations for a hypothetical case in which the protein species formed in the fast and slow refolding reactions have 10-fold different binding constants for 2'CMP: the products of slow and fast refolding are assigned dissociation constants of 1.3×10^{-5} M, and 1.3×10^{-4}M, respectively, and the value of α_2 at saturating 2'CMP is chosen to be 0.390 (so that the observed and calculated values of α_2 agree at the highest concentration of 2'CMP used).

TABLE 3. *Comparison of the kinetics of the fast and slow refolding reactions measured by tyrosine groups (A_{286}) and by 2'CMP binding (A_{250})*

Temperature °C	λ = 286 nm			λ = 250 nm			$\Delta A_{286}/\Delta A_{250}$	
	$\bar{\tau}_2$*(msec)	τ_1†(sec)	α_2‡	$\bar{\tau}_2$*(msec)	τ_1†(sec)	α_2‡	Fast phase	Slow phase
32	62	14.7	0.20	58	14.8	0.21	1.03	1.09
32 No 2'CMP	65	14.2	0.22	—	—			
36	46	10.9	0.21	42	10.8	0.21	0.98	0.94
40	41	8.7	0.21	45	8.9	0.22	1.02	1.08
40 No 2'CMP	48	9.0	0.22	—	—			
43	45	7.9	0.21	48	8.1	0.20	1.19	1.10
47	96	8.4	0.20	85	8.1	0.21	1.07	1.14
47 No 2'CMP	90	8.0	0.21	—	—			
51	370	7.8	0.17	380	7.7	0.15	1.34	1.21
51 No 2'CMP	380	7.6	0.15	—	—			

Initial conditions: RNase A 9.2×10^{-5} M; 0.1 M $NaClO_4$, ≃0.01 M $HClO_4$, pH 2.0.
Final conditions: RNase = 4.6×10^{-5} M; 2'CMP = 7.5×10^{-5} M; 0.05 M cacodylate, 0.1 M $NaClO_4$, pH 5.8.
* An average relaxation time of the fast refolding reaction measured as the weight-average value of $1/\tau_i$ (23). † The relaxation time of the slow refolding reaction measured from a plot of ln [A(∞) − A(*t*)] versus time. ‡ The relative amplitude of the fast refolding reaction expressed as a fraction of the total absorbance change. The absorbance changes associated with the fast and slow reactions have been found by assuming that each reaction follows a single exponential curve.

RNase A formed by fast refolding has a normal binding constant for 2'CMP. We may determine if the proteins folded in the fast and slow refolding reactions have different binding constants for 2'CMP by measuring the relative amounts of 2'CMP bound as a function of 2'CMP concentration. The results (Table 2) show no difference over a 10-fold range of 2'-CMP concentration at 47°, in which the fraction of RNase A binding 2'CMP at equilibrium varies from 0.164 to 0.795. For comparison, we calculated the expected results when the protein refolded in the fast reaction is assigned a 10-fold lower affinity for 2'CMP than the final equilibrium value. The results (Table 2) show that such a difference in affinity would have been detected readily.

The presence of 2'CMP has no effect on the kinetics of refolding measured by tyrosine groups. Since the difference spectrum between the 2'CMP: RNase A complex (12) and the separated reactants is almost zero at 286 nm, where there is a large change in absorbance on refolding, we can use this wavelength to monitor refolding independently of 2'CMP binding. Measurements of refolding at 286 nm show the same kinetics whether or not 2'CMP is present (Table 3); this is true both of the relaxation times and amplitudes. Consequently it is unlikely that 2'CMP binds to the starting material for either the fast or slow refolding reaction.

The kinetics of refolding are the same when measured by tyrosine groups and by 2'CMP binding. Different kinetics of refolding might be observed by buried tyrosine groups and by 2'CMP binding for either of two reasons: the tyrosine groups occur in different regions of the molecule and also might be expected to change in absorbance upon partial refolding, whereas 2'CMP binds at a single site and might be expected to bind only when refolding is complete. Thus, it is interesting to find that the kinetics are exactly the same when measured by either probe (Table 3). No reaction is found by one probe that is not discerned by the other, and the relative amplitudes are the same for both probes. Moreover, the results show that the molar value of ΔA_{286} during refolding is the same in the fast and slow refolding reactions*.

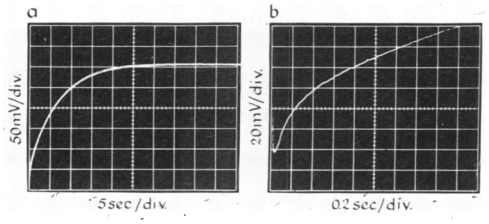

FIG. 3. Binding of 2'CMP occurs during both the fast and slow refolding reactions of RNase A (pH 2.0 → 5.8, 47°, 250 nm). Final concentrations: RNase A, 0.46×10^{-4}M; 2'CMP, 0.75×10^{-4}M. Total signal 2.2 V.

The protein folded in the fast reaction has enzymatic activity. The turnover time for RNase A to hydrolyze CpA giving C > p + A is fast [0.3 msec at 26° (16)] compared to both refolding reactions. Thus the corresponding change in A_{286} can be used as a fast probe of the recovery of enzymatic activity on refolding. (The enzyme concentrations used for these experiments are so low that one can ignore the change in A_{286} caused by RNase A refolding.) Fig. 4*b* shows that hydrolysis of CpA by native RNase A (pH 5.8), 47° produces a linear change with time for at least 2 sec, at specified concentrations of RNase A and CpA. In these conditions, the slope is also proportional to enzyme concentration (not shown). When the same stopped-flow experiment is repeated with an aliquot of RNase A that has been allowed to unfold previously at pH 2.0, 47°, and then to refold after a stopped-flow pH jump: 2.0 → 5.8 + CpA, the results shown in Fig. 4*a* are observed. The initial rate of CpA hydrolysis is small compared to that of the control (Fig. 4*b*) and the rate increases with time of refolding. The amount of enzymatic activity recovered at dif-

* This follows from that the fact that $\Delta A_{286}/\Delta A_{250}$ has the same value in the fast and slow refolding reactions (Table 3). Since the binding of 2'CMP to RNase A has a 1:1 stoichiometry, we may use ΔA_{250} and the equilibrium binding constant to determine the number of RNase A molecules refolded in a given reaction; thus $\Delta A_{286}/\Delta A_{250}$ is directly related to the molar change in absorbance at 286 nm.

TABLE 4. *Recovery of enzymatic activity during refolding**

Time (sec)	CpA hydrolysis†	Calc. (fast and slow)‡	Calc. (slow)§
0.25	0.06 ± 0.02	0.03	0.01
0.50	0.14 ± 0.02	0.09	0.02
0.75	0.22_5 ± 0.03	0.16	0.04
1.00	0.32 ± 0.03	0.24	0.08
1.25	0.41 ± 0.03	0.33	0.12
1.50	0.50 ± 0.04	0.43	0.17

* Conditions for RNase A refolding: 47°, pH 2.0 → 5.8 + CpA. Concentrations: CpA, 2×10^{-3} M; RNase A, 1.2×10^{-6} M. Silicon'zed glassware was used. † The amount of CpA hydrolyzed has been normalized by dividing by the initial rate of hydrolysis in the control with native RNase A; the units are sec^{1}. Let y be the amount of CpA hydrolyzed and c be the amount of active RNase A refolded at time t: then $y = k\int_0^t c\,dt$ and $y' = kc_\infty t$, where y' refers to the control and c_∞ is the amount of active RNase A after complete refolding (or before unfolding). Thus $y/(dy'/dt) = \int_0^t (c/c_\infty)dt$. In order to indicate the reproducibility of these measurements (which is better than their accuracy), the range of values found in 10 repeated measurements is shown. ‡ The fraction of active enzyme (c/c_∞) refolded at time t has been taken from kinetic data for 2'CMP binding, assuming that both the fast and slow refolding reactions yield active enzyme. Then the normalized amount of CpA hydrolyzed was predicted by numerical integration of $\int_0^t (c/c_\infty)dt$ (see footnote †). § Prediction of the normalized amount of CpA hydrolyzed on the assumption that only the slow refolding reaction yields active enzyme. See footnotes † and ‡.

ferent times, expressed in terms of the normalized amount of CpA hydrolyzed (Table 4), is somewhat larger than the amount predicted from the kinetics of refolding, on the assumption that both fast and slow refolding reactions yield active enzyme. We think that the difference is not significant, because the results of the CpA hydrolysis experiments depend sensitively both on the manner of cleaning the stopped-flow apparatus to remove traces of RNase A and on siliconizing the glassware to prevent losses of RNase A from dilute solutions. At early times of refolding, the amount of active enzyme recovered is much larger than predicted by the assumpthat only the slow refolding reaction yields active enzyme (Table 4). We conclude that active enzyme is formed in the fast refolding reaction.

The proportion of fast refolding at pH 5.8 is the same at temperatures inside and above the pH 2.0 transition zone. The fraction of protein refolding in the fast reaction, α_2, is essentially independent of temperature inside and above the pH 2.0 transition zone, provided that the temperature remains below the transition zone at the final pH of 5.8 (Table 3). If we suppose that the fast and slow refolding reactions arise from different starting materials, then the relative amounts of the fast and slow refolding species must be independent of temperature in this range. Thus, the fast refolding species cannot be a partly folded, nucleated species of the type discussed previously for a particular sequential model of folding (17).

CONCLUSIONS

(*1*) The fast reaction observed in the refolding of thermally unfolded RNase A does not represent the formation of an intermediate in refolding; instead, the product is fully active enzyme. It will be interesting to study other systems for which fast refolding reactions have been observed to see if this is a common phenomenon. (*2*) As judged by the behavior of tyrosine groups, the starting materials for the fast and slow refolding reactions have similar chromophoric properties, even though they show a 100-fold difference in their rates of refolding. (*3*) More experiments are needed before we can discuss models to represent these results: in particular, corresponding studies are needed of enzymatic activity during the fast phase of unfolding, and these data are not readily obtained by the techniques used here. However, we will comment briefly on three very simple models. (*a*) Two pathways of refolding: $U \underset{\text{slow}}{\overset{\text{fast}}{\rightleftharpoons}} N$. Here U is the thermally unfolded protein and N is native RNase. This model can be ruled out because it does not give biphasic kinetics (essentially all the molecules refold by the fast pathway). (*b*) Failure to obtain a completely unfolded starting material, because the temperature isn't high enough: $U \underset{\text{slow}}{\rightleftharpoons} N_1 \underset{\text{fast}}{\rightleftharpoons} N_2$. Here U and N_1 (a partly-folded species) are presumed to preexist in the initial conditions; N_1 is an intermediate present only in the thermal unfolding transition zone. This model is ruled out by the failure of N_1 to disappear at high temperatures, as discussed in the paragraph above. (*c*) Presence of two (or more) different unfolded forms: $U_1 \underset{\text{slow}}{\rightleftharpoons} U_2 \underset{\text{fast}}{\rightleftharpoons} N$. Here U_1 and U_2 are two different unfolded species that preexist in the initial conditions at all temperatures. They are linked by a common pathway of refolding, and are separated by a slow step. NMR measurements show a slow interconversion between native and thermally unfolded RNase A as well as what appear to be fast interconversions between native and partly unfolded species (18). Since there are reasons to believe that thermally unfolded RNase A retains some structure (19), the model has some *a priori* plausibility and it is able to explain most, but not all, of our present results.

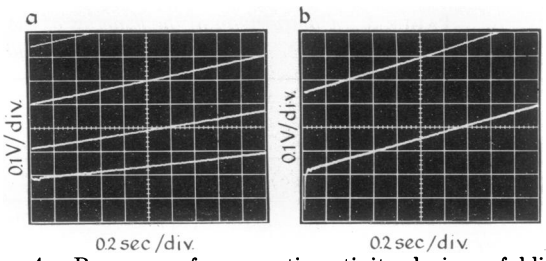

FIG. 4. Recovery of enzymatic activity during refolding of RNase A, as measured by hydrolysis of CpA at pH 5.8, 47°. Concentrations: CpA, 2×10^{-3} M; RNase A, 1.2×10^{-6}M. Wavelength, 286 nm, total signal 8 V. (*a*) Refolding measured by CpA hydrolysis after a pH jump: pH 2.0 → 5.8 + CpA. (*b*) A control at pH 5.8: hydrolysis of CpA by native RNase A.

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